African Journal of Biotechnology Vol. 9(15), pp. 2284-2289, 12 April, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Detection and identification of *Staphylococcus aureus* in raw milk by hybridization to oligonucleotide microarray

Yang He[1,2], Honglu Liu[3], Mengjun Xian[4] and Yufeng Li[2]*

[1]School of Medical Technology, Chengdu University of Traditional Chinese Medicine, 610041, Chengdu, China.
[2]School of Bioengineering, Xihua University, 610039, Chengdu, China.
[3]Sichuan Center for Disease Control and Prevention, 610041, Chengdu, China.
[4]College of Life Sciences, Sichuan University, 610064, Chengdu, China.

Accepted 1 February, 2010

Staphylococcus aureus **causes foodborne diseases if consumed in contaminated milk products. Rapid detection and characterization of foodborne pathogen *S. aureus* is crucial for epidemiological investigations and food safety surveillance. It is still a challenge to detect and identify bacterial pathogens quickly and accurately according to the samples. In this study, we have amplified 16S rRNA of *S. aureus* by specific primers, designed oligonucleotide probes, detected the sensitivity and specificity of the microarray assay, and also identified *S. aureus* in the raw milk samples by hybridization. The *S. aureus* and 2 control pathogens (*Streptococcus suis* in pigs and *Shigella*) were used for specificity of the microarray assay. Based upon the hybridization results, universal probes for bacterial pathogens, *S. aureus* probe, *Staphylococcus* spp. probe, nucleic acid fixture positive controls and positive experimental control showed positive signals with targeted *S. aureus*. The samples were diluted from 10^1 to 10^6 cfu per ml for evaluating the sensitivity of the microarray assay. The levels were as high as 10^3 cfu per ml, all of the samples showed positive signals. This method for rapid and effective detection and identification of *S. aureus* in raw milk demonstrated high sensitivity and specificity.**

Key words: Microarray, *Staphylococcus aureus*, raw milk.

INTRODUCTION

Milk is a nutritious food for human beings, acting as a good medium for the growth of many microorganisms, especially bacterial pathogens (Chye et al., 2004). *Staphylococcus aureus*, one of the most common bacterial pathogens by which raw milk is contaminated in most cases, can result in considerable negative effects on the quality of milk and dairy products. *S. aureus* is a facultative anaerobic gram-positive coccus, non-motile, catalase and coagulase positive which can be found in water, dust and air, but food handlers are the main sources of food contamination. Furthermore, *S. aureus* is an important human pathogen that causes foodborne infections in most countries of the world (Bergdoll, 1989), and also a wide spectrum of diseases ranging from benign skin infections to life-threatening endocarditis and toxic shock syndrome (Lowy, 1998; Projan and Novick, 1997). Staphylococcal food poisoning is mainly caused by the consumption of food containing staphylococcal enterotoxins (Ash, 1997). Raw milk is recommended to be pasteurized prior to consumption, *S. aureus* is effectively killed by pasteurization, but the enterotoxins produced by the *S. aureus* retained their biological activity even after pasteurization, which is becoming a hazard for consumers (Asao et al., 2003). Therefore, utilizing the microarray to assay the safety of pasteurized milk would be

*Corresponding author. E-mail: bioliyufeng@yahoo.cn. Tel: +86 28 87720549. Fax: +86 28 87720552.

Abbreviations: ***S. aureus*****,** *Staphylococcus aureus*; ***S. suis,*** *Streptococcus suis;* **LB,** Luria Bertani; **PCR,** polymerase chain reaction; **ssDNA,** single strand DNA; **rRNA,** ribosomal RNA; **SNRs,** signal-to-noise ratios.

Table 1. Pathogenic bacteria used in the study.

No.	Pathogens	Source
1	*Staphylococcus aureus* ATCC6538	Sichuan Center for Disease Control and Prevention
2	*Staphylococcus aureus*	Isolated from raw milk
3	*Streptococcus suis* in pigs	Sichuan Center for Disease Control and Prevention
4	*Shigella*	Sichuan Center for Disease Control and Prevention

Table 2. Oligonucleotide probes used in study.

No.	Name	Sequences (5'-3')
1	Bacteria universal probe	GCTGCCTCCCGTAGGAGT
2	*Staphylococcus aureus* probe	CGGACGAGAAGCTTGCTTCT
3	*Staphylococcus* spp. probe	TCCTCCATATCTCTGCGCAT
4	Negative probe	GTTGCTTCTGGAATGAGTTTGCT
5	*Streptococcus suis* in pigs probe	TTAGGGGGTTTCCGCCCCTTAGT
6	*Shigella* probe	GTCCGGGCGATCCCCTCGGTAGTG

indispensable to detect *S. aureus* at all stages of food manufacturing.

To ensure the quality of raw milk, it is important to supply requisitely rapid and reliable detection methods for *S. aureus*. The traditional culture-based methods are cumbersome and time-consuming (Aslam et al., 2003). Therefore, novel methods of milk safety are needed for fast and efficient detection of low numbers of *S. aureus* likely to be present in raw milk. Microarray technologies are powerful tools that can be used for the detection of multiple genes or target sequences on a single glass slide. The microarray is therefore a very useful device for detection of bacteria in food samples (Myers et al., 2006; Ikeda et al., 2006a; Ikeda et al., 2006b). This is due to their potential to discriminate and identify sequences of different origin rapidly and effectively (Kristensen et al., 2007). Recently, more studies have been performed to detect bacterium's presence mixed with different food matrices by way of the microarray (Siddique et al., 2009).

The aim of the present study is to develop a microarray method for convenient and rapid detection and identification of *S. aureus* in raw milk. In this study, we have designed oligonucleotide probes to analyse the sensitivity and specificity of the microarray assay. This method for easy and rapid detection and identification of *S. aureus* in raw milk is accurate and convenient.

MATERIALS AND METHODS

Bacterial strains, culture conditions and DNA extraction

Bacterial strains, as show in Table 1, were used in this study. Bacteria were inoculated into 50 ml liquid LB (Tryptone 10 g/l; Yeast Extract 5 g/l; NaCl 10 g/l) media, and grown 24 h at 37℃ on a shaker. Genomic DNA from a bacterial strain was prepared from 5 ml of a fresh overnight culture grown in LB at 37℃ by using a DNA extraction kit (TIANGEN, Beijing, China.) according to the manufacturer's instructions.

PCR primers and specific oligonucleotide probes

For *S. aureus*, *Streptococcus suis* in pigs and *Shigella*, the simplex PCR reaction conditions were: 1 µl of DNA template, 5 µl 10×PCR buffer, 2 mM $MgCl_2$, 1 µl dNTPs (each 2.5 mM), 0.4 µl each primer (50 µM) and 0.4 µl Taq DNA polymerase (5 U/µl), using the following procedures: 4 min at 94℃ and 32 cycles of 30 s at 94℃, 30 s at 58℃, 1 min at 72℃ and a final extension of 5 min at 72℃. The amplified bands were subjected to a 0.7% agarose gel electrophoresis, and examined under UV.

16S rRNA of *S. aureus* was amplified by newly designed specific primers (forward primer: 5`-GGTTTCGGATGTTACAGCGTAGAG TTTGATCCTGGCTCAG-3`, reverse primer: 5`-AAGGAGGTGAT CCAGCC-3`). Individual specific oligonucleotide probes were designed based upon the unique sequences found in the target genes using the ArrayDesigner software (Premier Biosoft International, Palo Alto, CA) (Table 2). The specificity of designed oligoprobes was assessed by using a BLAST search of homologous sequences available in the GenBank. The amplified 16S rRNA was labeled with fluorescent Cy3 according to the protocol described by Li et al. (2007).

Design and fabrication of oligonucleotide microarrys

Bacterial universal probes, *S. aureus* probe, *Staphylococcus* spp. probe, nucleic acid fixture positive and negative controls, *S. suis* in pigs probe, *Shigella* probe and experiment positive and negative controls were spotted on glass slides by the manufacturer (Boao, Beijing, China). The array described here included a total of 10 probes (Figure 1).

Microchips were prepared using a contact microspotting robotic system, PixSys 5000 (Cartesian Technologies, Inc., Irvine, CA, USA). The average size of the spots was 100 - 150 µm. The concentration of each oligoprobe before printing was adjusted to a

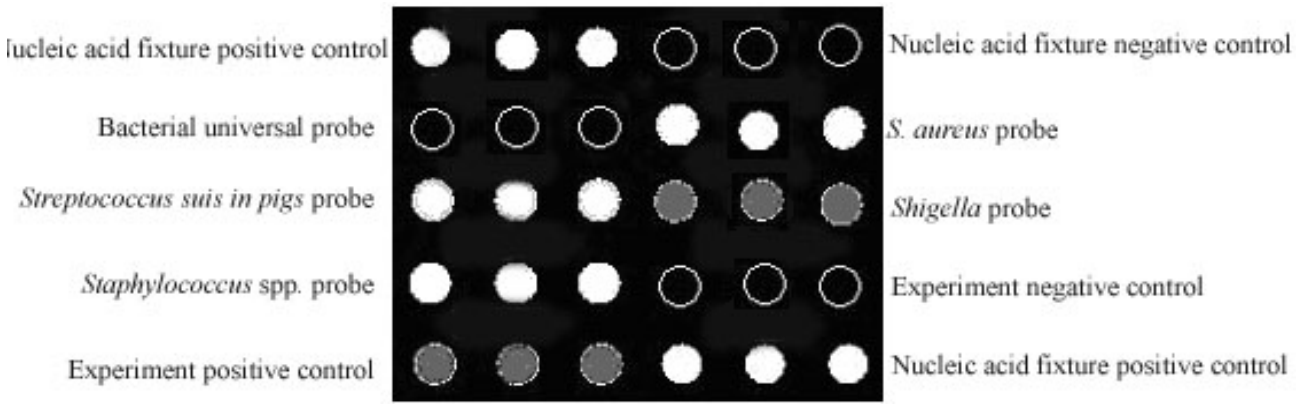

Figure 1. The design of oligonucleotide microarray. 10 probes were spotted on glass slide.

total of 100 µM in a 50% printing buffer (300 mM sodium phosphate, pH 8.5: H_2O).

Arrayed slides were prepared before hybridization according to the supplier's protocol with the exception of an additional prehybridization step with 5×SSC, 1% BSA, 0.1% SDS at 50°C for 1 h, followed by rinsing three times with sterilized distilled water for 1 min each. About 30 µl of the fluorescently labelled ssDNA sample was placed onto each of the two cells of the slide, then covered by a 5×5 mm plastic coverslip and incubated overnight (50°C) in a hybridization chamber (Proteigene, Saint Marcel, France). Coverslips were gently removed (2×SSC, 0.2% SDS, 5 min at 50°C) and slides were washed at room temperature in 10 successive washes: 2×SSC, 0.2% SDS twice for 5 min, 2×SSC three times for 3 min, 0.2×SSC for 5 min, 0.2×SSC three times for 2 min, 0.05×SSC for few seconds, and dried by centrifugation.

Microarray hybridization and scanning

All samples were hybridized in duplicate. Cy3-labeled PCR products were diluted in a total of 250 µl of Agilent hybridization buffer and hybridized at 60°C for 17 h in a dedicated hybridization oven (Robbins Scientific, Sunnyvale, CA). Slides were washed, dried under nitrogen flow, and scanned.

Fluorescent images were recorded with a GMS 418 fluorescent scanner (Affymetrix, Santa Clara, USA) and quantitatively analyzed by using the ImaGene 4.0 software (BioDiscovery, Inc., Los Angeles, CA). Signal-to-noise ratios (SNRs) were determined for each probe as Loy et al. (2002). Probe spots with SNRs equal to or greater than 2.0 were considered as positive.

Oligonucleotide microarray detection of *Staphylococcus aureus* in raw milk

S. aureus was enumerated by standard spread-plate method in duplicate. Authentic samples of raw milk were obtained from a collection tank of a local dairy farm. Preparation of artificially contaminated raw milk samples were carried out. For this purpose, the samples were inoculated with a colony of *S. aureus* which then were inoculated into 20 ml raw milk after pasteurized and incubated for 24 - 48 h at 37°C. The pasteurized raw milk was considered sterile after screening for *S. aureus* contamination according to the procedure described by Wang (2003). In the experiments with naturally or artificially contaminated samples, a total of 46 raw milk samples (16 naturally and 30 artificially contaminated samples) were examined by the microarray.

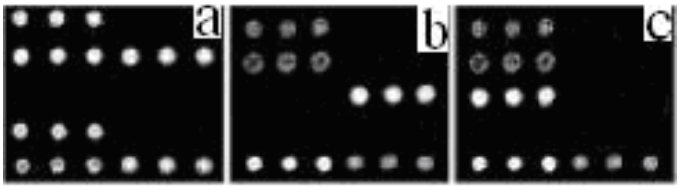

Figure 2. Pictures of (a) *S. aureus*, (b) *Shigella* and (c) *Streptococcus suis* in pigs. Specificity hybridization spot showed in respective hybridization results. At the same time, no non-specificity hybridization spot was shown. *S. aureus* and 2 pathogen controls demonstrated high specificity.

RESULTS AND DISCUSSION

Specificity of the microarray

To assess the functionality and specificity of the oligoprobes and eliminate cross-hybridization between pathogens affecting the final result, *S. aureus* and 2 control pathogens (*S. suis* in pigs and *Shigella*) were amplified with single PCR. The amplified PCR of each pathogen was used as a template in primer extinction reaction to create ssDNA. Ultimately, the ssDNA of each pathogen was labeled and hybridized separately on the microarray. Figure 2a shows the specificities of the oligoprobes for *S. aureus*.

Because of its specific nucleotide composition and the number, position, and types of mismatches to non-target organisms, theoretically, each individual probe on the microarray would require specific hybridization conditions to ensure its optimal specificity (Behr et al., 2000; Liu et al., 2001). Initially, the optimal washing temperature was determined experimentally as the best compromise between signal intensity and stringency for some of the probes by hybridizing the microarray with fluorescently labeled target DNA of *S. aureus* under increasing stringencies (data not shown). All following experiments were performed at the optimal washing temperature of 49°C. Subsequently, specificities of all probes were

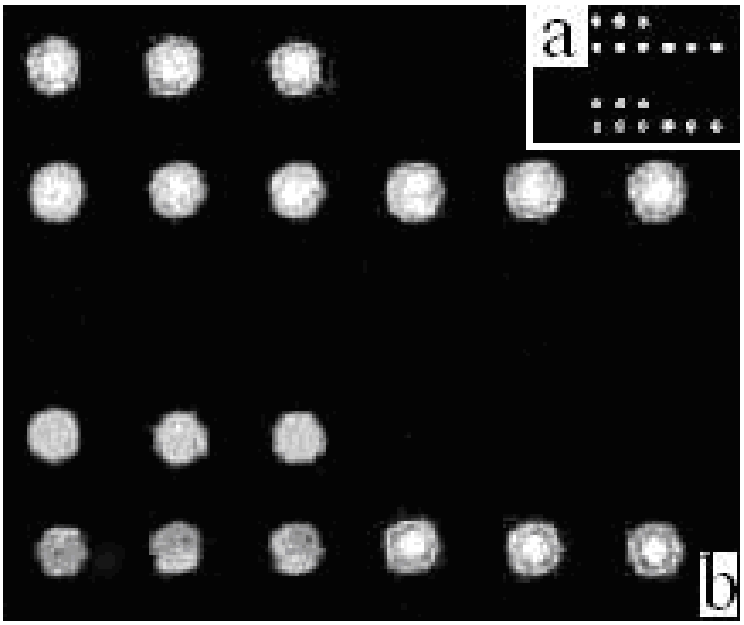

Figure 3. Microarray for detection and identification of *S. aureus* in raw milk samples. (a) Overview of microarray with samples. (b) Details showing hybridization results of samples. Bacterial universal probe, *S. aureus* probe, *Staphylococcus* spp. probe, nucleic acid fixture positive controls and experiment positive control showed positive signals. The other probes gave negative signals.

Table 3. Sensitivity test results for contaminated raw milk samples.

Strain	Test results					
	10^6 CFU/ml	10^5 CFU/ml	10^4 CFU/ml	10^3 CFU/ml	10^2 CFU/ml	10^1 CFU/ml
S. aureus	+	+	+	+	±	-

+= Positive signal; -= negative signal; ±= weak or ambiguous signal.

evaluated by hybridizing fluorescently labeled target DNA from each of the *S. aureus* and 2 control pathogens (*S. suis* in pigs *and Shigella*) hybridized with microarray. Based on the hybridization results (Figure 2), bacterial universal probe, *S. aureus* probe, *Staphylococcus* spp. probe, nucleic acid fixture positive controls and experiment positive control showed positive signals with target *S. aureus*. Hybridization with control gram-positive *S. suis in pigs*, bacterial universal probe, *S. suis* in pigs probe, nucleic acid fixture positive controls and experiment positive control demonstrated positive signals; with control gram- negative *Shigella*, bacterial universal probe, *Shigella* probe, nucleic acid fixture positive controls and experiment positive control exhibited positive signals.

Microarray analysed of raw milk samples

The ability to correctly identify *S. aureus* in naturally or artificially contaminated raw milk samples by microarray hybridization was determined. From the results of hybridizing with *S. aureus*, the universal probe, *S. aureus* probe, *Staphylococcus* spp. probe, nucleic acid fixture positive controls and positive experimental control showed positive signals (Figure 3). It is proved that this system can be used to detect and identify *S. aureus* in raw milk. Up to now, traditional methods and many methods in the fields of molecular biology have been used for detecting *S. aureus* (Schuenck et al., 2006; Hwang et al., 2007; Leeuwen et al., 2003; Graber et al., 2007; Huang and Chang, 2004). At the same time, the microarray method has been modified and applied in several aspects (Call et al., 2003; Gentry et al., 2006; Hong et al., 2004; Jin et al., 2007; Kostić et al., 2007). In comparison to the traditional method and other molecular methods, the microarray method is high throughput, specific, and sensitive and also avoids most cross-

reactions.

Sensitivity of microarray detection

Naturally or artificially, contaminated raw milk samples were diluted from 10^1 to 10^6 cfu per ml, and then tested with the microarray. When the levels were higher than 10^3 cfu per ml, all of the samples showed positive signals; at a lower level, as low as 10^2 cfu per ml, all of the samples showed weak or ambiguous signals. However, when the level was at 10^1 cfu per ml, all of the samples showed negative signals (Table 3). So, in our study, the limit of detection (LOD) reached 10^3 cfu per ml. To date, microarray detection of pathogens have been performed in food materials (Siddique et al., 2009). Furthermore, these systems had almost sensitivities reaching the level of 10^3 or 10^4 cfu per ml (Myers et al., 2006; Li et al., 2006). Moreover, a step of PCR amplification is included before the hybridization, and this step ensures that low concentrations of specific pathogen and deteriorated bacteria are detected in the dairy product as reported by Call et al. (2003).

Conclusion

In the search for fast and simple genetic techniques, oligo-nucleotide microarray has gained acceptance in the methods of detecting *S. aureus* because it is high through put, specific, sensitive and easy to perform. Our research was pursuing and developing a kind of microarray method for detection and identification of *S. aureus* in raw milk. In this study, 16S rRNA of *S. aureus* was amplified by newly designed specific primers, and oligonucleotide probes was designed. This was used to analyse the sensitivity and specificity of the microarray assay. *S. aureus* and 2 control pathogens (*S. suis* in pigs and *Shigella*) were used for specific detection through microarray. The hybridization results illustrated that bacterial universal probe, *S. aureus* probe, *Staphylococcus* spp. probe, nucleic acid fixture positive controls and a positive control in the experiment showed positive signals with target *S. aureus*. The sensi- tivity of the microarray assay was high, and it reached 10^3 cfu per ml.

The microarray system with high throughput and low cost developed in this study can provide powerful and additional evidence to traditional methods for more accurate risk assessment and monitoring of *S. aureus* in raw milk.

ACKNOWLEDGMENTS

This work was supported by the grants from the Fund of Chengdu University of Traditional Chinese Medicine (no. ZRYB200949) and the key project of Sichuan Province (no. 04JY029-053-1 and no. R0520503).

REFERENCES

Asao T, Kumeda Y, Kawai T, Shibata T, Oda H, Haruki K, Nakazawa H, Kozyki S (2003). An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. Epidemiol. Infect. 130: 3-40.

Ash M (1997). *Staphylococcus aureus* and Staphylococcal nEnterotoxins. In: Hocking, A.D.; Arnold, G.; Jenson, I.; Newton, K.; Sutherland, P. ed. Foodborne Microorganisms of Public Health Significance. North Sydney, Aust. Institute Food Sci. Technol. Inc. pp. 313-332.

Aslam M, Hogan J, Larry Smith K (2003). Development of a PCR-based assay to detect Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* in milk. Food Microbiol. 20: 45-350.

Behr T, Koob C, Schedl M, Mehlen A, Meier H, Knopp D, Frahm E, Obst U, Schleifer K, Niessner R, Ludwig W (2000). A nested array of rRNA targeted probes for the detection and identification of enterococci by reverse hybridization. Syst. Appl. Microbiol. 23: 563-572.

Bergdoll MS (1989). *Staphylococcus aureus* In: Doyle MP(ed) Food borne Bacterial Pathogens, Marcel Dekper, Inc: New York. pp. 463-523.

Call DR, Borucki MK, Loge FJ (2003). Detection of bacterial pathogens in environmental samples using DNA microarrays. J. Microbiol. Meth. 53: 235-243.

Chye FY, Abdullah A, Ayob MK (2004). Bacteriological quality and safety of raw milk in Malaysia. Food Microbiol. 21: 535-541.

Gentry TJ, Wickham GS, Schadt CW, He Z, Zhou J (2006). Microarray applications in microbial ecology research. Microb. Ecol. 52: 159-175.

Graber HU, Casey MG, Naskova J, Steiner A, Schaeren W (2007). Development of a Highly Sensitive and Specific Assay to Detect *Staphylococcus aureus* in Bovine Mastitic Milk. J. Dairy Sci. 90: 4661-4669.

Hong BX, Jiang LF, Hu YS, Fang DY, Guo HY (2004). Application of oligonucleotide array technology for the rapid detection of pathogenic bacteria of foodborne infections. J. Microbiol. Meth. 58: 403-411.

Huang SH, Chang TC (2004). Detection of *Staphylococcus aureus* by a Sensitive Immuno-PCR Assay. Clin. Chem. 50: 1673-1674.

Hwang SY, Kim SH, Jang EJ, Kwon NH, Park YK, Koo HC, Jung WK, Kim JM, Park YH (2007). Novel multiplex PCR for the detection of the *Staphylococcus aureus* superantigen and its application to raw meat isolates in Korea. Int. J. Food Microbiol. 117: 99-105.

Ikeda M, Yamaguchi N, Tani K, Nasu M (2006a). Detection of Food Poisoning Bacteria in Fresh Vegetables Using DNA Microbiology. J. Health Sci. 52 (1): 36-42.

Ikeda M, Yamaguchi N, Tani K, Nasu M (2006b). Rapid and simple detection of food poisoning bacteria by bead assay with a microfluidic chip-based system. J. Microbiol. Meth. 67(2): 241-247.

Jin DZ, Xu XJ, Chen SH, Wen SY, Ma XE, Zhang Z, Lin F, Wang SQ (2007). Detection and identification of enterohemorrhagic *Escherichia coli* O157:H7 and *Vibrio cholerae* O139 using oligonucleotide microarray. Infect. Agent. Cancer 2: 23.

Kostić T,Weilharter A, Rubino S, Delogu G, Uzzau S, Rudi K, Sessitsch A, Bodrossy L (2007). A microbial diagnostic microarray technique for the sensitive detection and identification of pathogenic bacteria in a background of nonpathogens. Anal. Biochem. 360: 244-254.

Kristensen R, Gauthier G, Berdal KG, Hamels S, Remacle J, Holst-Jensen A (2007). DNA microarray to detect and identify trichothecene- and moniliformin-producing *Fusarium* species. J. Appl. Microbiol. 102: 1060-1070.

Leeuwen WB, Jay C, Snijders S, Durin N, Lacroix B, Verbrugh HA, Enright MC, Troesch A, Belkum A (2003). Multilocus Sequence Typing of *Staphylococcus aureus* with DNA Array Technology. J. Clin. Microbiol. 41: 3323-3326.

Li Y, Liu D, Cao B, Han W, Liu Y, Liu F, Guo X, Bastin DA, Feng L, Wang L (2006). Development of a Serotype-Specific DNA Microarray for Identification of Some *Shigella* and Pathogenic *Escherichia coli*

Strains. J. Clin. Microbiol. 44: 4376-4383.
Liu WT, Mirzabekov AD, Stahl DA (2001). Optimization of an oligonucleotide microchip for microbial identification studies: A non-equilibrium dissociation approach. Environ. Microbiol. 3: 619-629.
Lowy FD (1998). *Staphylococcus aureus* infections. N. Engl. J. Med. 339: 520-532.
Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, Schleifer KH, Wagner M (2002). Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. 68: 5064-5081.
Myers KM, Gaba J, Al-Khaldi SF (2006). Molecular identification of *Yersinia* enterocolitica isolated from pasteurized whole milk using DNA microarray chip hybridization. Mol. Cell. Probe. 20(2): 71-80.
Projan S, Novick R (1997). The molecular basis of pathogenicity. *In* G. Archer and K. Crossley (ed.), Staphylococci in human diseases. Churchill Livingstone, New York. pp. 55-81.
Schuenck RP, S.Lourenco MC, L.P.Iório N, P.Ferreira AL, A.Nouér S, N.Santos KR (2006). Improved and rapid detection of methicillin-resistant *Staphylococcus aureus* nasal carriage using selective broth and multiplex PCR. Res. Microbiol. 157: 971-975.
Siddique N, Sharma D, Al-Khaldi SF (2009). Detection of *Yersinia enterocolitica* in Alfalfa, Mung Bean, Cilantro, and Mamey Sapote (*Pouteria sapota*) Food Matrices Using DNA Microarray Chip Hybridization. Curr. Microbiol. 59: 233-239.
Wang CE (2003). Labortary manual of medical microbiology. Zhongshan University Press, Guangzhou, Guangdong. pp. 41-51.